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Enzyme Immunoassay of Human Urinary Kallikrein

Determination of human urinary kallikrein, III.[1])

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Summary: An enzyme immunoassay for the determination of human urinary kallikrein has been developed and is compared with other human urinary kallikrein assays such as radioimmunoassay, dog blood pressure assay, rat uterus test after kinin liberation and synthetic substrate[2]) tests (AcPheArgOEt and S-2266). The usable range of the standard curve is from 0.05 to 12 ng kallikrein per test. The intraassay coefficient of variation is between 2 and 4%, the interassay coefficient of variation is between 4 and 12%, and the recovery of authentic kallikrein added to urine samples is 95%. Human saliva and human pancreatic kallikrein show the same binding curves as purified human urinary kallikrein. Kallikrein from urine of rats, dogs and rabbits as well as boar acrosin and pig pancreatic kallikrein, bovine trypsin and chymotrypsin show no cross-reactivity.

Enzymimmunoassay für menschliches Harnkallikrein
Bestimmung von menschlichem Harnkallikrein, III

Zusammenfassung: Ein Enzymimmunoassay zur Bestimmung menschlichen Harnkallikreins wurde entwickelt und mit anderen Harnkallikrein-Bestimmungsmethoden wie Radioimmunoassay, Hundeblutdruck-Test, Rattenuterustest nach Kininfreisetzung und Aktivitätstest mit synthetischen Substraten[2]) (AcPheArgOEt und S-2266) verglichen. Der zur Bestimmung von Harnkallikrein verwendbare Bereich auf der Standardkurve lag zwischen 0,05 und 12 ng pro Test. Der Intraassaykoeffizient lag zwischen 2 und 4%, der Interassaykoeffizient zwischen 4 und 12% und die Wiederfindungsrate von Harnkallikrein, das zu Harnen zugegeben wurde, bei 95%. Menschlicher Speichel und Pankreas-Kallikrein ergaben Bindungskurven, die mit der für reines Harnkallikrein erhaltenen Bindungskurve identisch waren. Kallikrein aus Harn von Ratten, Hunden und Kaninchen, Schweine-Akrosin und Schweinepankreas-Kallikrein sowie Trypsin und Chymotrypsin des Rindes zeigten keine Kreuzreaktion.

Introduction

Determination of tissue kallikrein (EC 3.4.21.35) in human urine and body fluids is of special interest because of its postulated physiological role in kidney function and blood pressure regulation, fertilization processes, glucose metabolism and intestinal absorption (1, 2).

Various methods for the determination of urinary kallikrein are available at present. Some assays such as the biological assay (reduction of dog blood pressure after intravenous urine injection (3)) and the kinin-liberating test (kallikrein-induced kinin liberation from kininogens and determination of the kinins by biological assays, immunoassays or high performance liquid chromatography (4)) are cumbersome and not suitable for routine purposes. Other methods based on the hydrolysis of synthetic substrates, e.g. N^{α}-benzoyl-*L*-arginine ethyl ester, N^{α}-tosyl-*L*-arginine methyl ester, AcPheArgOEt and S-2266[2]) are often not specific and sensitive enough for kallikrein determination in tissue extracts or other body fluids. In addition, they do not allow the measurement of kallikrein in the presence of inhibitors i.e. in the form of the enzyme-inhibitor complex (5–10).

[1]) I. = l.c. (23); II. = l.c. (11)

[2]) Abbreviations:

AcPheArgOEt:	N^{α}-acetyl-*L*-phenylalanyl-*L*-arginine ethyl ester
DValLeuArgNHNp:	*D*-valyl-*L*-leucyl-*L*-arginine-*p*-nitroanilide (= S-2266 from Kabi)
ABTS	2,2'-azino-bis (3-ethyl-benzthiazoline-6-sulfonate)

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Recently, a radioimmunoassay has been developed for the determination of human urinary kallikrein (11) and since that time this assay has become a common method for the determination of human tissue kallikrein. However, general problems of the radioimmunoassay such as special and expensive equipment for radioactive measurements and the disposal of radioactive waste stimulated the development of alternative but also highly sensitive immunoassays. This report describes an enzyme immunoassay for human tissue kallikrein allowing estimation of kallikrein levels as low as 0.5 µg/l in a reasonably short time (5 hours incubation time).

Material and Methods

Human urinary kallikrein, used as a standard in the enzyme immunoassay, was purified as described (12). Human pancreatic kallikrein was isolated as described by *Hofmann* et al. (13). Horseradish peroxidase (EC 1.11.1.7; grade 1) and bovine trypsin and chymotrypsin were products of Boehringer, Mannheim. Boar acrosin was kindly provided by *Müller-Esterl & Fritz* (14), human high molecular weight kininogen by *Dittmann* et al. (15). Pig pancreatic kallikrein was a gift of Bayer AG, Wuppertal. Bovine serum albumin was purchased from Behringwerke AG, Marburg/Lahn. Bradykinin was a product of Bachem AG, Bubendorf, Switzerland. AcPheArgOEt[2]) was synthesized as described by *Fiedler* et al. (8). DValLeuArg-NHNp[2]) was purchased from Kabi AB, Mölndal, Sweden. Tween 20 was a product of Serva AG. Microtiter plates M 129 B were purchased from Dynatech, Denkendorf, West-Germany.

Antibody production in rabbits was performed as described by *Mann* et al. (11). IgG fractions were isolated according to *Steinbuch & Audran* (16).

Sample collection

24-hour urine samples of male and female healthy volunteers were collected. The urines were dialysed against running water for 24 h. Saliva obtained by selective catherization of the glandular duct was collected before and after oral administration of 2 ml citric acid (100 g/l), freeze-dried and stored at – 20 °C. Pancreatic juice was collected by a duodenal catheter in a X-ray monitored position. Samples of serum and seminal plasma were obtained from healthy volunteers.

Buffers

Buffer A: 15 mmol/l Na_2CO_3, 350 mmol/l $NaHCO_3$, 0.2 g/l NaN_3, pH 9.6.

Buffer B: 1.5 mmol/l KH_2PO_4, 8 mmol/l Na_2HPO_4, 2.7 mmol/l KCl, 150 mmol/l NaCl, 0.5 g/l, Tween 20, pH 7.4.

Buffer C: 1.5 mmol/l KH_2PO_4, 8 mmol/l Na_2HPO_4, 2.7 mmol/l KCl, 150 mmol/l NaCl, 0.5 g/l, Tween 20, 20 g/l bovine serum albumin, pH 7.4.

Buffer D: 100 mmol/l citric acid, 100 mmol/l K_2HPO_4, pH 4.0.

Kallikrein activity

Human kallikrein activity was measured using DValLeuArg-NHNp and AcPheArgOEt as substrates (17). Kallikrein-induced kinin liberation from human urinary kallikrein by radioimmunoassay was performed according to *Mann* et al. (11).

Biological assay

Kinin activity was assayed by the rat uterus assay as described by *Mann* et al. (10). Bradykinin served as reference for kinin activity determination. Dog blood pressure assay was performed as described by *Frey* et al. (3).

Coupling of peroxidase to IgG

Preparation of peroxidase IgG conjugate was performed as described by *Nakane & Kawoi* (18). After gel filtration on Sephadex G-100 the anti-human urinary kallikrein-IgG peroxidase conjugate was diluted 1:50 with buffer C before being used for the enzyme immunoassay.

Enzyme immunoassay conditions

Microtiter plates were coated with the same anti-human urinary kallikrein-IgG solution (IgG 10 mg/l buffer) (0.2 ml per well) as used for the peroxidase IgG conjugate at 4 °C over night. The plates were then washed vigorously (3 times) in buffer B. Human urinary kallikrein standard samples and test samples were diluted with buffer C. Of these samples 0.2 ml were added to the wells and the plates were incubated at 37 °C for 3 h in order to bind the kallikrein to the solid-phase fixed antibodies. After incubation, the plates were washed (3 times) with buffer B, 0.2 ml of the anti-human urinary kallikrein-IgG peroxidase conjugate was added to each well, and the plates were incubated again at 37 °C for 2 h. After washing peroxidase activity was measured as described by *Anaokar* et al. (19) by addition of 0.2 ml working substrate solution (stock substrate consisted of 200 mg ABTS[2]) dissolved in 10 ml water). To prepare working substrate, 0.5 ml stock soln. was added to 9.5 ml buffer D and 0.2 ml H_2O_2 solution (H_2O_2, 300 g/kg, diluted 0.6 ml/l) was added and the plates were incubated for 30 min at room temperature. Thereafter, the reaction was stopped by addition of 0.05 ml sodium azide (10 g/l). The absorption was read photometrically at 405 nm with a Microelisa®-reader AM 115 from Dynatech, Denkendorf, West-Germany. Background generally ranged from $A_{405\,nm}$ = 0.02 to 0.08. In each test a standard concentration curve with purified human urinary kallikrein was included. Standard curves were constructed by plotting the fraction of absorbance at 405 nm, $A - N/A_0 - N$ (A: absorbance, A_0: maximal absorbance obtained with excess human urinary kallikrein, N: absorbance of the blank) against the dose of human urinary kallikrein in a logit-log mode or in a linear-log mode.

A scheme of the incubation conditions is shown in Table 1.

Tab. 1. Scheme of human urinary kallikrein (HUK) enzyme immunoassay. Samples were in triplicate.

	Sample (ml)	Blank (ml)
Anti-HUK-IgG (10 mg/l)	0.20 15 h at 4 °C	0.20
Standard or sample	0.20	–
Buffer C	– 3 h at 37 °C	0.20
Anti-HUK-IgG peroxidase conjugate, dil. 1:50	0.20 2 h at 37 °C	0.20
Substrate solution	0.20 30 min at 22 °C	0.20
Sodium azide (10 g/l)	0.05	0.05
	absorbance read at 405 nm	

Results

Concentration of the solid-phase IgG

The least amount of IgG required to coat the microtiter plates was determined from standard curves of data obtained with plates treated with concentrations

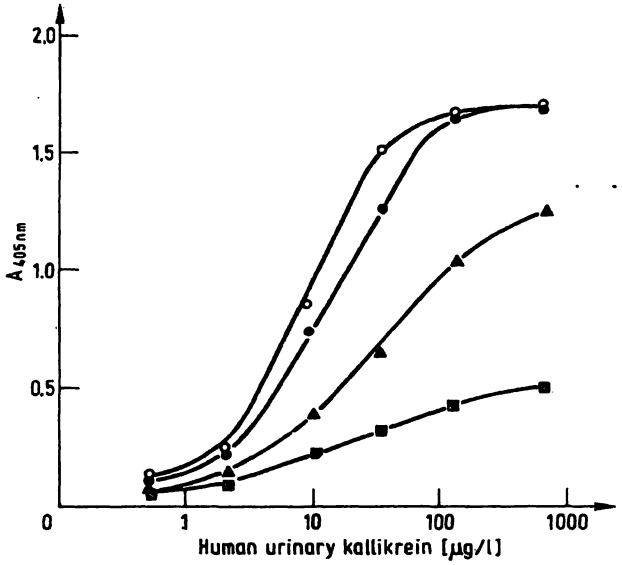

Fig. 1. Evaluation of the optimal concentration of the solid-phase antibody. Dose response curves for human urinary kallikrein obtained with microtiter plate wells coated with 1 mg (■—■), 2.5 mg (▲—▲), 5 mg (●—●) and 10 mg (○—○) of anti-human urinary kallikrein-IgG per liter.

of IgG ranging from 1 to 10 mg/l. As seen in figure 1, the standard curves were identical for IgG concentrations between 10 and 5 mg/l whereas standard curves obtained with IgG concentrations lower then 5 mg/l showed a decreased binding of human urinary kallikrein. Hence, we decided to use an IgG concentration of 10 mg/l for the assay. Anti-human urinary kallikrein-IgG peroxidase conjugate dilution in the experiment was fixed at a 50-fold dilution.

Anti-human urinary kallikrein-IgG peroxidase conjugate dilution

The optimal dilution of the conjugate was determined from a standard curve obtained with microtiter plates coated with an IgG solution of 10 mg/l and conjugate diluted 100-, 50-, 20- and 10-fold with buffer C. As the 50-fold dilution was the highest dilution of the conjugate at which optimal absorbance at 405 nm occurred, this conjugate dilution was chosen for the assay.

Time and temperature dependence of the antigen antibody reactions

To determine the optimal incubation conditions for the enzyme immunoassay the time and temperature dependence of the first incubation, the binding of human urinary kallikrein to the solid-phase fixed antibody, was studied at 22 and 37 °C. The highest binding rate was observed at 37 °C, where equilibrium was reached after 3 hours (fig. 2a). The optimal time and temperature required for the second reaction, binding of anti-human urinary kallikrein-IgG peroxidase conjugate to solid-phase immunglobulin-fixed human urinary kallikrein, was similarily determined. As shown in figure 2b, this time equilibrium was reached after 2 hours at 37 °C. Concentration of the IgG-solution used for coating was 10 mg/l, and the anti-human urinary kallikrein-IgG peroxidase conjugate dilution was 20-fold.

Standard curves and sensitivity

The standard curve was consistently linear for human urinary kallikrein concentrations between 0.25 and 60 µg/l under routine conditions (fig. 3). The sensitivity of an enzyme immunoassay can be defined as "the smallest amount of antigen giving a response which is distinguishable from the response in the absence of antigen" (20). The lowest concentration of human urinary kallikrein that produced a response greater than that caused in the absence of human urinary kallikrein was 0.25 µg/l, which corresponds to 50 pg per well.

Precision

The intraassay coefficient of variation (N = 10) was 2–4% for human urinary kallikrein (range 0.25 to 64 µg/l). The interassay coefficient of variation of identical samples (N = 10) containing 1, 4, 16, 64 µg/l was 4–12%.

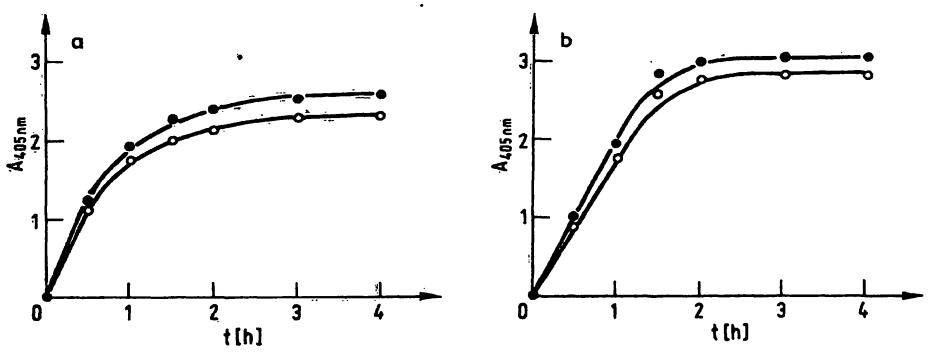

Fig. 2. Incubation time and temperature dependence of the first (a) and second (b) reaction. Dose response curves for human urinary kallikrein were obtained by incubating standard (10 µg/l) at room temperature (○) and 37 °C (●).

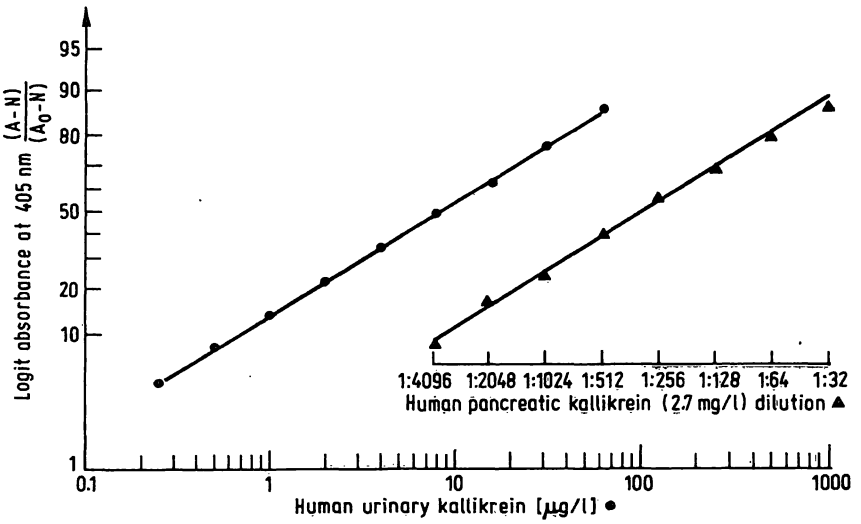

Fig. 3. Dose response curves of purified human urinary kallikrein and human pancreatic kallikrein in the human urinary kallikrein enzyme immunoassay, ●—●, standard human urinary kallikrein, ▲—▲, human pancreatic kallikrein (2.7 mg/l).

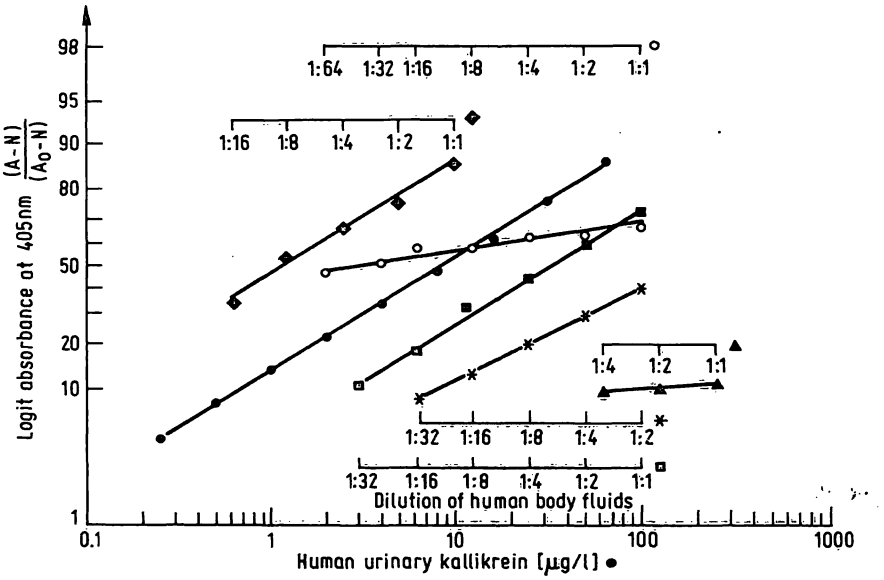

Fig. 4. Dose response curves of human urinary kallikrein and human body fluids in the human urinary kallikrein enzyme immunoassay; ●—●, standard human urinary kallikrein, ○—○, pancreatic juice, ■—■, saliva (500 µg/l), ◆—◆, urine, ▲—▲, serum, *—*, seminal plasma.

Specificity

Cross-reaction of other serine proteinases with anti-human urinary kallikrein-IgG was tested under the given immunoassay conditions. A cross-reaction was not observed with bovine trypsin and chymotrypsin, pig pancreatic kallikrein, boar acrosin and urine from rat, dog and rabbit. In the human samples investigated, immunologically active material was found in pancreatic juice, saliva, urine, serum and seminal plasma. Suitable dose-response curves are shown in figure 4.

Recovery test

Recovery tests in which three different quantities of purified human urinary kallikrein were added to antigen-free rat urine showed a good precision of the assay. The data are listed in table 2.

Tab. 2. Recovery test.

Human urinary kallikrein			
Added (µg/l)	Recovered (µg/l)	N	Recovery (%)
20	19.0 ± 0.46	10	95 ± 2.3
10	10.5 ± 0.12	10	105 ± 1.2
5	5.1 ± 0.06	10	102 ± 1.2

Stability of the microtiter plates coated with anti-human urinary kallikrein-IgG

Microtiter plates coated with anti-human urinary kallikrein-IgG were air-dried and stored at 4 °C in sealed plastic bags containing silica gel. Identical standard curves were obtained under routine conditions within a period of 3 weeks after coating. Thereafter a decrease of binding was observed.

Comparison of the enzyme immunoassay with other methods

In order to show the correlation of the enzyme immunoassay with other determination methods for human urinary kallikrein, 10 dialysed urine test samples were measured by different kallikrein assays. As can be seen from figure 5 the correlation coefficients of the synthetic substrate assays with AcPheArgOEt and DValLeuArgNHNp, the dog blood pressure assay, the kinin-liberation assay, the radioimmunoassay of human urinary kallikrein and the enzyme immunoassay are close to 1.

Tab. 3. Comparison of the enzyme immunoassay for human urinary kallikrein with other assays for human urinary kallikrein. 10 samples of dialysed urine were measured by each method.

Assays for human urinary kallikrein	Correlation coefficients obtained with human urinary kallikrein enzyme immunoassay
Radioimmunoassay (11)	0.9932
Dog blood pressure (3)	0.9643
Kinin liberation (10)	0.9824
Synthetic substrates	
AcPheArgOEt (17)	0.9910
DValLeuArgNHNp (17)	0.9920

Discussion

The sensitive and specific enzyme immunoassay developed for human urinary kallikrein should be especially suitable for further studies on the physiological and pathophysiological role of tissue kallikreins. As the different tissue kallikreins in humans are immunologically indistinguishable (21), this assay can be used to detect all of them.

The high specificy of the enzyme immunoassay could be demonstrated by employing various serine proteinases. Cross reactivity was not observed with bovine trypsin and chymotrypsin, boar acrosin and pig pancreatic kallikrein. Furthermore, in analogy to the results obtained by radioimmunoassay of human urinary kallikrein (11) we may expect that human proteinases other than tissue kallikreins do not interfere in the enzyme immunoassay.

Dose response curves obtained with immunologically cross-reacting material from pancreatic juice, serum and seminal plasma were not parallel with each other. These results are in agreement with data obtained using the radioimmunoassay for human urinary kallikrein (11), except for the binding curves for human pancreatic juice. This is of interest since purified human pancreatic kallikrein will give parallel curves in the enzyme immunoassay for human urinary kallikrein. Explanations for these interferences could be the presence of:

1) prokallikrein or modified kallikrein with immunologically different properties,

2) other, as yet uncharacterized, cross-reacting antigens, and

3) inhibitors in the samples which reduce binding to antibody due to kallikrein-inhibitor complex formation.

Further nonspecific interferences cannot be excluded at present. All these questions need to be further investigated and methods for the elimination interference must be developed.

A number of experiments were undertaken to compare the enzyme immunoassay with assays established already for the determination of human urinary kallikrein. For this purpose dialysed urine samples from 10 different persons were employed. The results obtained with the enzyme immunoassay were compared with those obtained by the dog blood pressure assay, a radioimmunoassay for human urinary kallikrein, a rat uterus assay after kallikrein-induced kinin liberation, and assays with AcPheArgOEt and DValLeuArgNHNp as kallikrein substrates. The correlation between the synthetic substrate assays, the rat uterus assay after kallikrein-induced kinin liberation and the radioimmunoassay is more satisfactory than the correlation obtained by comparison of dog blood pressure assay and enzyme immunoassay. Though the correlation coefficient of the results of the enzyme immunoassay and the dog blood pressure assay is rather close to 1, a considerable scattering of the data is observed. This is not too surprising, since the coefficient of variation of the blood pressure assay for kallikrein is approximately 20% (22). The excellent correlation, however between the two immunoassays indicates the high validity of the enzyme immunoassay.

Considerable advantages of the enzyme immunoassay over the radioimmunoassay are, among others, the lack of radiation hazards, greater stability of the label, higher throughput and ease of automation. The assay procedure is simple to perform in a relatively short time and requires only small quantities of kallikrein. The minimal detectable dose is 50 pg per well. Therefore, this assay offers a precise and feasible test for human urinary kallikrein, which can be used as an alternative to the radioimmunoassay.

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